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# SecA is an intrinsic subunit of the *Escherichia coli* preprotein translocase and exposes its carboxyl terminus to the periplasm

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## Summary

**SecA is the dissociable ATPase subunit of the *Escherichia coli* preprotein translocase, and cycles in a nucleotide-modulated manner between the cytosol and the membrane. Overproduction of the integral subunits of the translocase, the SecY, SecE and SecG polypeptides, results in an increased level of membrane-bound SecA. This fraction of SecA is firmly associated with the membrane as it is resistant to extraction with the chaotropic agent urea, and appears to be anchored by SecYEG rather than by lipids. Topology analysis of this membrane-associated form of SecA indicates that it exposes a carboxy-terminal domain to the periplasmic face of the membrane.**

## Introduction

Export of proteins across the inner membrane of *Escherichia coli* is catalysed by the preprotein translocase. The preprotein translocase (Brundage *et al.*, 1990) consists of the integral membrane subunits SecY (Ito, 1992), SecE (Schatz *et al.*, 1989, Matsuyama *et al.*, 1990) and SecG (Nishiyama *et al.*, 1993, 1994) and the peripheral subunit SecA (Schmidt *et al.*, 1988; Hartl *et al.*, 1990; Oliver, 1993). Together these proteins suffice for the energy-dependent translocation of preproteins into reconstituted proteoliposomes (Brundage *et al.*, 1990; Douville *et al.*, 1994; Nishiyama *et al.*, 1993; 1994; Hanada *et al.*, 1994). The homodimeric SecA recognizes the signal-sequence domain and targets the preprotein to the membrane, where it interacts with negatively charged phospholipids

and the SecYEG complex. A subset of preproteins first associates with the export-dedicated chaperone SecB (Kumamoto and Nault, 1989; Lecker *et al.*, 1989) before being targeted to SecA (Hartl *et al.*, 1990; Hoffschulte *et al.*, 1994). SecA is a preprotein-stimulated ATPase (Lill *et al.*, 1989) and undergoes nucleotide-modulated cycles of membrane insertion and de-insertion (Breukink *et al.*, 1992; Van der Wolk *et al.*, 1993; Economou and Wickner, 1994). It uses the energy of ATP binding to insert into the cytoplasmic membrane and to release the signal-sequence domain of the bound preprotein (Schiebel *et al.*, 1991; Economou and Wickner, 1994). The interaction between SecA and the SecYEG complex activates SecA for ATP hydrolysis (Lill *et al.*, 1989; Brundage *et al.*, 1990; Hartl *et al.*, 1990). This process drives the release of the preprotein from SecA and promotes de-insertion of SecA from the cytoplasmic membrane. Preproteins may then be stepwise-translocated across the membrane (Schiebel *et al.*, 1991) through co-insertion with SecA (Economou and Wickner, 1994).

A photo-cross-linking approach that employed a stable protein translocation intermediate was used to show that SecA and SecY, but not SecE or phospholipid, are in the environment of the translocating chain of the preprotein (Joly and Wickner, 1993). This suggests that the membrane-integrated form of SecA associates with SecY to form a protein-conducting channel. A 30 kDa SecA fragment was found to be resistant to proteolytic digestion of *E. coli* inside-out (ISO) membrane vesicles during *in vitro* preprotein translocation in the presence of ATP, and it has been suggested that this domain penetrates the membrane to expose a region to the periplasmic face of the membrane (Economou and Wickner, 1994). Another study, however, suggests that even without translocation, SecA may completely traverse the membrane and expose domains to the periplasmic face of the membrane (Kim *et al.*, 1994). Some cellular SecA is intimately associated with the cytoplasmic membrane (Cabelli *et al.*, 1991) and can be only partly removed by treating the membranes with urea (Cunningham *et al.*, 1989; Watanabe and Blobel, 1993). It is not known which part or domain of SecA associates with, or is inserted into, the membrane. It is also not obvious what relationship exists between the membrane-inserted state of SecA under translocating (Economou

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**Table 1.** Bacterial strains and plasmids.

Strain/Plasmid	Relevant characteristic	Reference/Source
<i>E. coli</i> strain		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Hanahan (1983)
JM105	<i>supE</i> <i>endA</i> <i>sbcB15</i> <i>hsdR4</i> <i>rpsL</i> <i>thi</i> $\Delta$ ( <i>lac-proAB</i> )	Yanisch-Perron <i>et al.</i> (1985)
NO2947	$\Delta$ <i>lac</i> [I $\phi$ OZYA], <i>araD139</i> , $\Delta$ [ <i>ara-leu</i> ]7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> , <i>recA56</i> <i>srl::Tn10</i> , <i>r<sub>k</sub>m<sub>k</sub></i>	K. Linton
BL21(DE3)	<i>hsdS</i> <i>gal</i> ( $\lambda$ .c1ts857 <i>ind1</i> <i>sam7</i> <i>nin5</i> <i>lacUV5</i> - <i>T7</i> gene 1	Studier and Moffatt (1986)
SF100	<i>F</i> <sup>-</sup> $\Delta$ <i>lacX74</i> <i>galE</i> <i>galK</i> <i>thi</i> <i>rpsL</i> ( <i>strA</i> ) $\Delta$ <i>phoA</i> ( <i>PvuII</i> ), $\Delta$ <i>ompT</i>	Bareyx and Georgiou (1990)
UT5600	<i>F</i> <sup>-</sup> <i>ara14</i> <i>leuB</i> <i>azi6</i> <i>lacY1</i> <i>proC14</i> <i>tsx67</i> ( $\Delta$ <i>ompT</i> - <i>fepC</i> )266 <i>entA403</i> <i>trpE38</i> <i>rfbD1</i> <i>rpsL109</i> <i>xyl5</i> <i>mtl1</i> <i>ompP</i>	Elish <i>et al.</i> (1988)
Plasmid		
pBlueIIISK+	amp <i>T7</i> promoter	Stratagene
pUC19	amp <i>lacZ</i>	Yanisch-Perron <i>et al.</i> (1985)
pTRC99A	amp <i>trc</i> promoter	Amann <i>et al.</i> (1988)
pET301	pTRC99A with <i>NcoI</i> - <i>Bam</i> HI fragment ( <i>secE</i> ) of pET316	This work
pET313	pSK(II)+ with <i>KpnI</i> - <i>Clal</i> PCR fragment of <i>secY</i>	This work
pET314	pSK(II)+ with <i>Bam</i> HI- <i>XbaI</i> PCR fragment of <i>secG</i>	This work
pET316	pSK(II)+ with <i>Clal</i> - <i>Bam</i> HI PCR fragment of <i>secE</i>	This work
pET324	<i>NarI</i> (B)- <i>Bam</i> HI fragment of pUC19 cloned into the <i>PstI</i> (B)- <i>Bam</i> HI site of pTRC99A	This work
pET329	pSK(II)+ with <i>SalI</i> - <i>Clal</i> PCR fragment of <i>secY</i>	This work
pET335	<i>SalI</i> - <i>XbaI</i> fragment of pET329 containing <i>secY</i> cloned into pET313	This work
pET338	<i>Clal</i> - <i>Bam</i> HI fragment of pET316 cloned into pET347 ( <i>T7</i> , <i>secYEG</i> )	This work
pET340	<i>NcoI</i> - <i>XbaI</i> fragment of pET338 cloned into pET324 ( <i>trc</i> , <i>secYEG</i> )	This work
pET347	<i>Bam</i> HI- <i>XbaI</i> fragment of pET314 cloned into pET335 ( <i>T7</i> , <i>secYEG</i> )	This work

(B) indicates that the site is made blunt using T4 polymerase.

and Wickner, 1994) and non-translocating (Kim *et al.*, 1994) conditions.

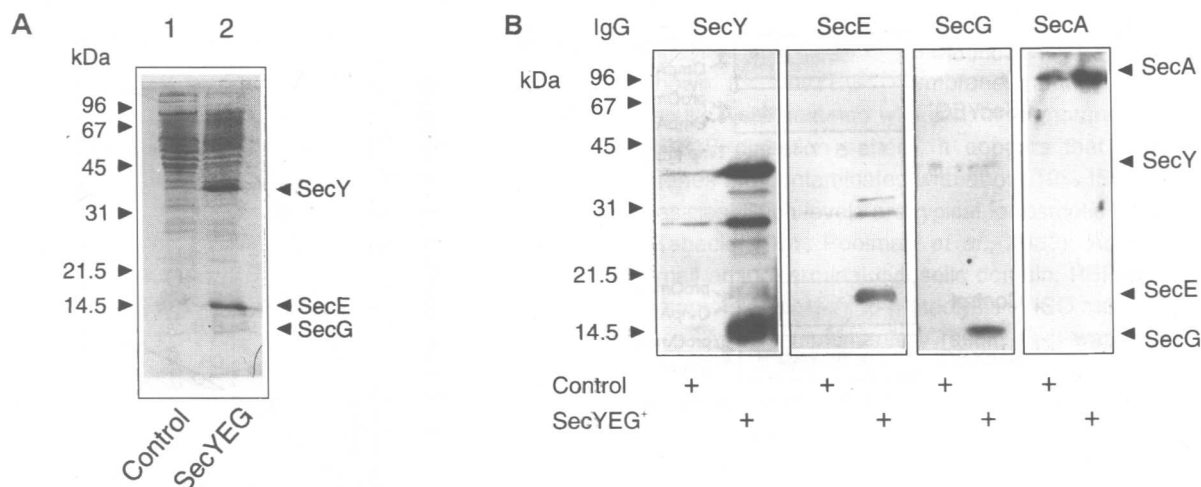
We now show that the high-level, plasmid-directed overexpression of the heterotrimeric integral membrane domain of the translocase results in an increased level of SecA membrane association. This form of SecA is active in translocation and appears to be firmly attached to the membrane while it exposes a carboxy-terminal domain to the periplasmic face of the membrane under non-translocating conditions. It is concluded that SecA is an intrinsic membrane-protruding subunit of the translocase, and the possible role of membrane anchoring is discussed.

## Results

### Overexpression of SecY, SecE and SecG

To achieve overexpression of the integral subunits of the preprotein translocase, a synthetic operon was created with

the gene order *secY*-*secE*-*secG*, and placed behind the inducible *T7* and *trc* promoters (Table 1). Inducible overexpression of a *secE*-*secY*-*secG* construct has been reported by Douville *et al.* (1995). Different combinations of strains and plasmids were checked under inducing conditions for expression levels of the SecY, SecE and SecG proteins by SDS-PAGE (Fig. 1A) and immunoblotting (Fig. 1B) of isolated membranes. Although with all strains and constructs substantial overexpression of SecY, SecE and SecG could be achieved, the highest levels were obtained with the *ompT* deletion strain SF100 transformed with a vector harbouring the *secYEG* synthetic operon under the control of the inducible *trc* promoter (pET340) (Fig. 1). Based on quantification of Coomassie brilliant blue-stained gels, the SecY polypeptide represents at least 15% of the membrane proteins in this strain. Further experiments were performed with membranes derived from SF100 cells harbouring either pET340 (SecYEG<sup>+</sup>) or pET324 (control).



**Fig. 1.** Overexpression of the SecY, SecE, and SecG proteins in *E. coli* SF100 cells.

A. Coomassie brilliant blue-stained SDS-PAGE of membranes derived from SF100 cells harbouring plasmids pET324 (control) (lane 1) or pET340 (SecYEG<sup>+</sup>) (lane 2). The positions of the molecular mass markers are indicated.

B. Immunoblots of control and SecYEG<sup>+</sup> membranes developed with pAbs raised against SecA and synthetic peptides corresponding to SecY, SecE, and SecG domains. Anti-SecY pAb stains, in addition to intact SecY, two amino-terminal degradation products of SecY with apparent molecular masses of about 24 and 14.5 kDa.

#### Functional characterization of SecYEG<sup>+</sup> membrane vesicles

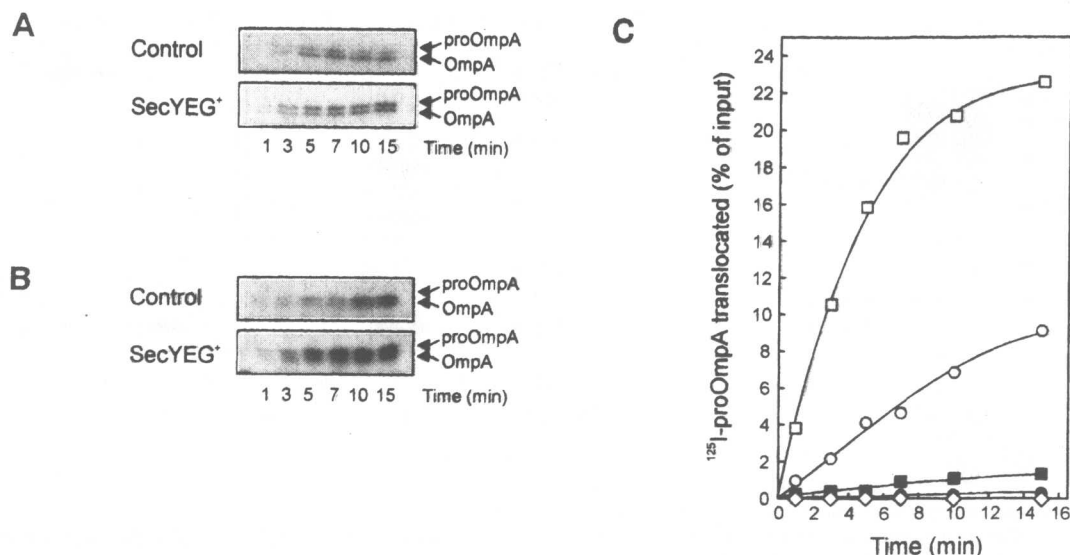
To establish whether the SecYEG complex was functionally overexpressed, the preprotein-stimulated translocation ATPase and translocation activity of ISO membrane vesicles derived from SecYEG<sup>+</sup> and control cells was determined. Membranes were extracted with 6 M urea to inactivate the membrane-resident SecA (Cunningham *et al.*, 1989). Even after this treatment, a significant level of translocation ATPase activity was detected in the SecYEG<sup>+</sup> membranes, i.e.  $50 \pm 7$  nmol of released P<sub>i</sub> mg<sup>-1</sup> of protein min<sup>-1</sup> ( $n=9$ ). Addition of purified SecA dramatically stimulated the translocation ATPase activity to a level of 380 nmol mg<sup>-1</sup> protein min<sup>-1</sup>. The translocation ATPase activity of control membranes was negligible in the absence of added SecA ( $<2$  nmol mg<sup>-1</sup> protein min<sup>-1</sup>) and increased to about 80 nmol mg<sup>-1</sup> protein min<sup>-1</sup> in the presence of SecA.

To preclude the notion that translocation is limited by the availability of preprotein, saturating amounts of [<sup>125</sup>I]-labelled proOmpA were used to assay for translocation, rather than *in vitro*-synthesized [<sup>35</sup>S]-proOmpA. In the presence of an excess of purified SecA, SecYEG<sup>+</sup> membrane vesicles showed an enhanced (four- to fivefold) rate of [<sup>125</sup>I]-proOmpA translocation (Fig. 2, B and C). Even in the absence of added SecA, a low but significant level of ATP-dependent proOmpA translocation was observed with the urea-treated SecYEG<sup>+</sup> membranes (Fig. 2C). As expected (Cabelli *et al.*, 1988; Cunningham *et al.*, 1989), a strict requirement for SecA addition was not evident when non-urea-treated membranes were used (Fig. 2A),

while addition of an excess of purified SecA resulted in a twofold stimulation of translocation (data not shown). In control membranes, a greater fraction of the translocated proOmpA was processed to OmpA as compared to SecYEG<sup>+</sup> membranes (Fig. 2A), suggesting that the amount of leader peptidase in SecYEG<sup>+</sup> membranes is a limiting factor in processing. These data demonstrate that the SecYEG complex is overproduced in a functional state.

#### Overexpression of SecYEG increases the amount of SecA that is tightly associated with the membrane

The total amount of SecA in both control and SecYEG<sup>+</sup> cells was determined by quantitative immunoblotting using a dilution series. SecYEG<sup>+</sup> cells consistently contained about  $1.3 \pm 0.1$  ( $n=3$ ) times the amount of total SecA found in the control cells. To examine the cellular distribution of SecA, extracts obtained by sonication were separated into a soluble (cytosolic) fraction and a pellet (membrane) fraction by ultracentrifugation analogous to the method described by Cabelli *et al.* (1991). SecYEG<sup>+</sup> membranes contained a larger amount of membrane-bound SecA than the control membranes (Fig. 3, A and B). The increase in SecA membrane association was accompanied with a slight decrease of the SecA present in the cytosolic fraction. Sucrose-gradient-purified SecYEG<sup>+</sup> membranes used for the translocation assays shown in Fig. 2B bear about three- to fourfold more SecA than the control membranes (Figs 1B and 3B). When membranes were extracted with 6 M urea, nearly all of the SecA was removed from the control membranes, while in SecYEG<sup>+</sup>



**Fig. 2.** Overexpressed SecYEG complex is active in preprotein translocation. The time-course of the translocation of  $^{125}\text{I}$ -labelled proOmpA into native (A) and urea-extracted (B) ISO membrane vesicles derived from control and SecYEG<sup>+</sup> cells is shown. Quantification of the translocation of proOmpA into urea-extracted membranes derived from control (circles) and SecYEG<sup>+</sup> cells (squares) (C) was assessed in the absence (closed symbols) and presence (open symbols) of  $40\ \mu\text{g}\ \text{ml}^{-1}$  of SecA. Open diamond symbols: the translocation of proOmpA into urea-treated SecYEG<sup>+</sup> membranes in the presence of SecA and in the absence of ATP.

membranes only a fraction was removed (Fig. 3B). A second extraction of these membranes with urea did not further reduce the amount of membrane-bound SecA (data not shown). Taken together, these results indicate that upon SecYEG-overproduction, a greater share of the cellular SecA is membrane-associated, consistent with the observation that SecYEG overexpression increases the number of high-affinity SecA-binding sites (Douville *et al.*, 1995). A considerable portion of the SecA bound to SecYEG<sup>+</sup> membranes, however, resists urea-extraction, suggesting that this subfraction of SecA is tightly associated with the integral domain of the translocase.

#### *Increased membrane association of SecA is not due to a changed phospholipid content*

The level of SecA membrane association is known to vary with the phospholipid composition of the membrane (Lill *et al.*, 1990; Rietveld *et al.*, 1995). To exclude the possibility that the extra SecA membrane binding is due to an altered phospholipid content in cells overexpressing SecYEG, the phospholipid composition of membranes derived from the SecYEG<sup>+</sup> and control cells was determined. No significant difference was detected in the amount of chloroform/methanol-extractable phospholipids per mg of cellular protein between both cell types (Table 2). The total amount of acidic phospholipids (cardiolipin and phosphatidylglycerol) increased only slightly from 27 to 33 mol% in the SecYEG-overproducing strain (Table 2). Since the substitution of all of the phosphatidylethanolamine for acidic

phospholipids causes only a twofold increase in the amount of membrane-associated SecA (Rietveld *et al.*, 1995), it is concluded that the moderate change in phospholipid composition in the SecYEG-overproducing strain cannot account for the large increase in membrane-bound SecA.

#### *SecYEG-associated SecA penetrates the membrane and exposes a domain to the periplasmic face of the membrane*

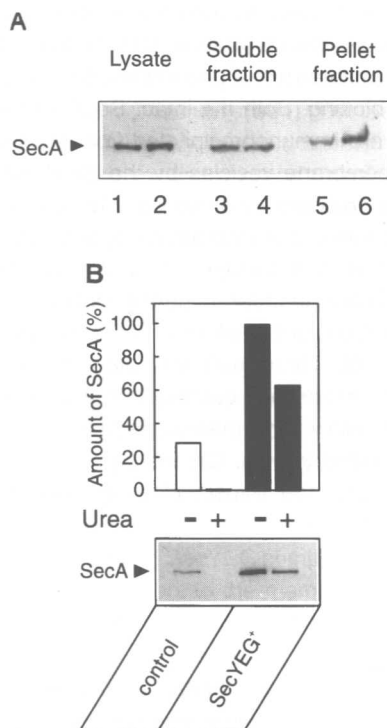
To determine if the SecYEG-bound SecA is in a membrane-inserted state, the trypsin-susceptibility of SecA was analysed in rightside-out (RSO) membrane vesicles

**Table 2.** Phospholipid composition of the parental and SecYEG overproducing *E. coli* strain SF100.

Phospholipid	Control pET324	SecYEG <sup>+</sup> pET340
	% <sup>a</sup>	
Phosphatidylethanolamine	73	67
Phosphatidylglycerol	22	21
Cardiolipin	5	12
	nmol P <sub>i</sub> mg <sup>-1</sup> protein <sup>b</sup>	
Phospholipid:protein ratio	1.26	1.23

**a.** Phospholipid composition is expressed in mol% on the basis of phosphate content and set to 100% for the three dominant lipid species. The remainder of the phospholipids, i.e. less than 1%, was lysophosphatidylethanolamine.

**b.** Amount of chloroform/methanol-extractable phospholipid per mg of cell protein.



**Fig. 3.** Overproduction of the SecYEG complex increases the amount of membrane-associated SecA.

**A.** The subcellular location of SecA in control and SecYEG<sup>+</sup> cells is shown. Cell lysates (lane 1, control; lane 2, SecYEG<sup>+</sup>) were fractionated into a soluble (control, lane 3; SecYEG<sup>+</sup>, lane 4) and a pellet (control, lane 5; SecYEG<sup>+</sup>, lane 6) fraction by centrifugation. The pellet fraction was resuspended in the original volume of fresh buffer, and, for each fraction, identical volumes were loaded on SDS–PAGE, blotted and immunostained with an anti-SecA pAb. **B.** Urea-extraction of SecA from control and SecYEG<sup>+</sup> ISO vesicles. As indicated, membrane vesicles (0.5 mg of protein ml<sup>-1</sup>) in 50 mM Tris-HCl, pH 7.6, were incubated for 30 min at 4°C with or without 6 M urea. Membranes were subsequently collected by ultracentrifugation (TLA100, 200 000 × *g*, 60 min), washed once, and resuspended in identical volumes of 50 mM Tris-HCl, pH 7.6. Equal volumes of the various fractions (about 12 µg of protein for non-urea-treated membranes) were subjected to SDS–PAGE, and further analysed by immunoblotting with a pAb against SecA. The blot was quantified by densitometric scanning. The amount of SecA associated with non-urea-treated SecYEG<sup>+</sup> vesicles was set to 100%.

that expose the periplasmic membrane face to the outside, and in ISO membrane vesicles that expose the cytosolic face of the membrane to the outside. The correct 'sidedness' of these membranes was confirmed by analysing the trypsin-susceptibility of the  $\beta$ -subunit of the  $F_1$ -domain of the  $F_1F_0$ -ATPase as a marker of the cytosolic face of the membrane, and penicillin-binding protein 1B (PBP1B) as a marker of the periplasmic face of the membrane. PBP1B is a periplasmic protein that is anchored to the cytoplasmic membrane by an amino-terminal membrane-spanning segment (Nakagawa and Matsuhashi, 1982; den Blaauwen and Nanninga, 1990). ISO and RSO membrane vesicles were incubated for 15 min on ice (PBP1B) or 3 h at 30°C ( $F_1$ - $\beta$ ) with increasing trypsin concentrations and analysed

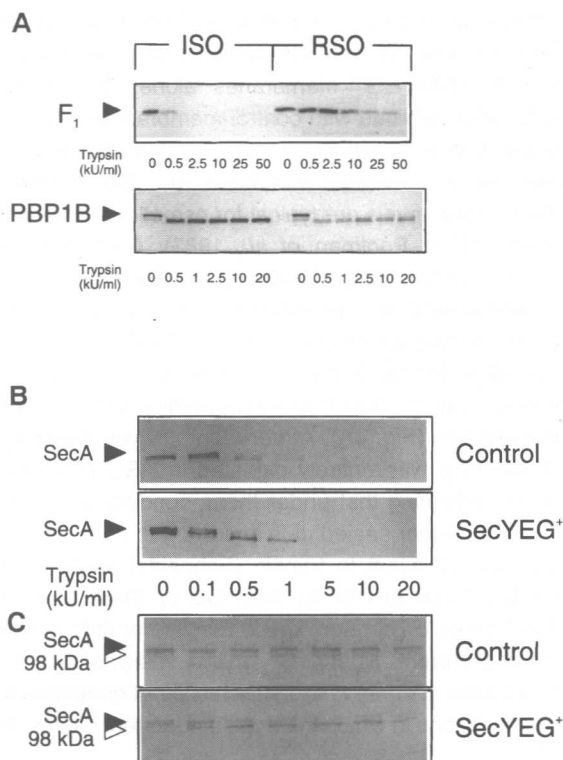
by SDS–PAGE and immunoblotting. PBP1B was readily digested in the RSO membrane vesicles (Fig. 4A, data shown for SecYEG<sup>+</sup> membranes alone, but identical results were obtained with control membranes). From the PBP1B digestion pattern, it appears that these membranes are contaminated with about 19% ISO membrane vesicles. Such levels are typical for osmotic lysis vesicles (Kaback, 1971; Poolman *et al.*, 1983). Apart from the small amino-terminal cytosolic domain, PBP1B is almost completely resistant to proteolysis in ISO membrane vesicles. As a complementary result,  $F_1$ - $\beta$  was found to be readily digested by trypsin in ISO membrane vesicles, whereas it was far less trypsin-susceptible in RSO membrane vesicles (Fig. 4A). At very high trypsin concentrations the  $F_1$ - $\beta$  was entirely digested in RSO membrane vesicles, indicating that under those conditions the vesicles are no longer sealed. It is important to note that  $F_1$ - $\beta$  is far more resistant to trypsin (Schneider *et al.*, 1995; Fig. 4A) relative to PBP1B (Fig. 4A) or SecA (Fig. 4B, and see below). Therefore these results not only demonstrate the correct 'sidedness' of the vesicles, but also show that the integrity of the vesicles is not compromised under conditions in which the trypsin treatment digests the SecA.

SecA was readily digested by trypsin in ISO membrane vesicles (Fig. 4B), consistent with the notion that SecA exposes large domains to the cytosolic side of the membrane. Free SecA was about 50-fold more sensitive to trypsin-digestion than was SecA bound to ISO membranes. On the other hand, in RSO membrane vesicles of the control only limited proteolysis of SecA occurred, and a subfraction was digested to a 98 kDa fragment that was not further proteolysed (Fig. 4C). Strikingly, almost all of the SecA in the SecYEG<sup>+</sup> RSO membrane vesicles was digested to the 98 kDa fragment, while only a small fraction of the SecA remained intact. Taking into account the fact that the samples were normalized for SecA protein prior to loading on the gel to correct for the increased level of SecA membrane association in SecYEG<sup>+</sup> membranes relative to the control (Fig. 3), the data demonstrate that in the SecYEG-overproducing strain a greater amount of membrane-bound SecA exposes a trypsin cleavage site to the periplasmic face of the membrane.

#### *The 98 kDa tryptic fragment of SecA lacks the carboxyl terminus*

The tryptic 98 kDa fragment found in RSO membrane vesicles represents either an amino-terminal or a carboxy-terminal truncate of SecA. To determine the identity of this fragment, a polyclonal antiserum (pAb) was raised against a synthetic peptide that corresponds to the carboxy-terminal 20 amino acids of SecA. In trypsin-treated control and SecYEG<sup>+</sup> RSO membrane vesicles, the antibody





**Fig. 4.** Trypsin-accessibility of SecA in ISO and RSO membrane vesicles of control and SecYEG<sup>+</sup> cells.

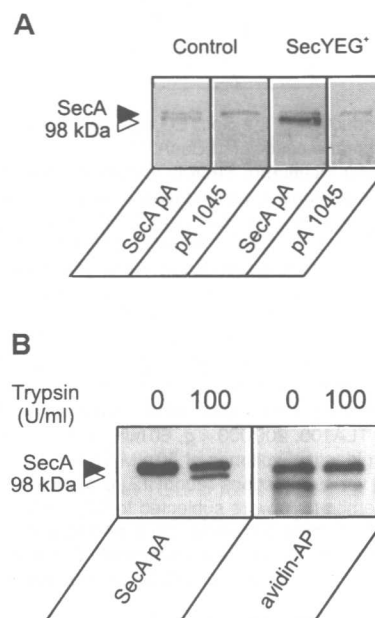
**A.** Trypsin digestion of the F<sub>1</sub>- $\beta$  subunit of the F<sub>1</sub>F<sub>0</sub>-ATPase (F<sub>1</sub>) (cytosolic membrane face marker) and penicillin-binding protein 1B (PBP1B) (periplasmic membrane face marker) in ISO and RSO membrane vesicles of SecYEG<sup>+</sup> cells. Membranes were incubated with trypsin, as described in the *Experimental procedures* section, and, after quenching of the proteolysis by the addition of trypsin inhibitor, the vesicles were analysed using 10% SDS-PAGE. Immunoblots of trypsin-treated ISO and RSO membrane vesicles were developed with pAb against F<sub>1</sub>- $\beta$  or a mAb F2-12 against PBP1B.

**B.** Trypsin digestion of SecA in ISO membrane vesicles of control and SecYEG<sup>+</sup> cells.

**C.** Trypsin digestion of SecA in RSO membrane vesicles of control and SecYEG<sup>+</sup> cells. Immunoblots in (B) and (C) were developed with a pAb against SecA. The open arrow indicates the tryptic 98 kDa SecA fragment formed in RSO membrane vesicles.

detected the intact SecA but failed to immunostain the tryptic 98 kDa fragment (Fig. 5A). Immunostaining of the same blot with a pAb against SecA confirms the presence of the intact SecA and the 98 kDa fragment (Fig. 5A). As an alternative approach, the carboxy-terminal cysteines of SecA were biotinylated with 3-(*N*-maleimidylpropionyl)biotin (MPB) (Bayer *et al.*, 1985). SecA has cysteinyl residues at amino-acid-residue positions 98, 885, 887, and 896, of which the cysteine at position 98 is poorly accessible for bulky maleimides (Driessen, 1993). RSO membrane vesicles of control cells were incubated with trypsin to convert a fraction of the SecA into the 98 kDa fragment. Although MPB is membrane permeable (Loo and Clarke, 1995), membranes were sonicated in addition

during the biotinylation to ensure complete equilibration of the MPB across the membrane. Subsequently, SecA was immunoprecipitated with a pAb against SecA and analysed by immunoblotting. Both the intact SecA and the 98 kDa fragment were immunoprecipitated from the trypsin-treated RSO membrane vesicles by the SecA pAb as both forms were detected by a mouse oAb (oligoclonal antibody, i.e. a mixture of monoclonal antibodies) (Fig. 5B). Staining of the same sample with avidin-conjugated alkaline phosphatase, in order to identify biotinylated proteins, stained only the intact SecA and not the 98 kDa SecA fragment (Fig. 5B). These data indicate that the SecYEG-bound SecA exposes a carboxy-terminal domain to the periplasmic face of the membrane that is accessible to externally added trypsin. On the basis of the loss of a 4 kDa fragment, the trypsin cleavage site is probably after Arg-850.



**Fig. 5.** The 98 kDa tryptic SecA fragment lacks its carboxyl terminus.

**A.** RSO membrane vesicles of control and SecYEG<sup>+</sup> cells were incubated with 110 U ml<sup>-1</sup> trypsin as described and analysed by immunoblotting using an anti-SecA pAb, and pAb 1045 was raised against a synthetic peptide that recognizes the carboxy-terminal 20 amino acid residues of SecA. The amount of membrane protein loaded on the gel was 8  $\mu$ g.

**B.** RSO membrane vesicles of control cells were incubated with 100 U ml<sup>-1</sup> trypsin, and biotinylated with MBP as described in the *Experimental procedures* section. SecA protein was immunoprecipitated with anti-SecA pAb, and analysed by immunoblotting using an oAb against SecA and a streptococcal avidin-alkaline phosphatase conjugate to detect the biotin. The tryptic 98 kDa SecA fragment is indicated by the open arrow. The additional band stained by avidin-APase is an unrelated protein that is co-immunoprecipitated by the anti-SecA pAb but not recognized by the oAb.

## Discussion

In recent years, compelling evidence has demonstrated that the *E. coli* preprotein translocase consists of the SecA, SecY, SecE and SecG proteins (for reviews, see Wickner *et al.*, 1991; Driessen, 1994). The SecA subunit is particularly intriguing as it is a preprotein-stimulated ATPase that exists in soluble and membrane-bound forms (Cabelli *et al.*, 1991), while the membrane-bound form may alternate between a surface-bound and membrane-integrated state (Breukink *et al.*, 1992; Economou and Wickner, 1994). The functional overexpression of the SecY, SecE, and SecG proteins from a tandem gene construct has been reported before (Douville *et al.*, 1995), but the impact of the overexpression on the SecA-membrane association and topology has not been studied. We now show that in a SecYEG-overproducing strain, the level of membrane-bound SecA is elevated, most of the protein being firmly associated with the membrane. The increased membrane binding at the SecYEG complex is due to a shift of the cytosolic SecA pool to the membrane-bound form and a slight increase in the total amount of cellular SecA. Further examination of the SecA membrane topology demonstrates that the protein exposes a carboxy-terminal region to the periplasmic face of the membrane. It is concluded that SecA is a membrane-protruding subunit of the translocase, even under conditions in which it is not involved in protein translocation.

SecA binds with low affinity to phospholipids (Hendrick and Wickner, 1991), and with high affinity ( $K_d \approx 40$  nM) to the SecYEG complex (Hartl *et al.*, 1990). To observe high-affinity SecA binding (Hartl *et al.*, 1990) and translocation that strictly depends on the addition of purified SecA (Cunningham *et al.*, 1989), membrane vesicles must be extracted with urea. This treatment only partly removes the SecA from the membrane (Cabelli *et al.*, 1991; Watanabe and Blobel, 1993; this study), while it has been argued that the urea-resistant SecA retains the ability to translocate preproteins (Watanabe and Blobel, 1993). SecYEG overproduction results in an increased number of SecA high-affinity binding sites in urea-extracted membranes (Douville *et al.*, 1995). Our study elaborates on this observation and demonstrates that the overproduction of the SecYEG complex elevates the amount of membrane-bound SecA without producing a notable change in the lipid composition. Most of the additionally bound SecA cannot be extracted by 6 M urea or by 100 mM sodium hydrogen carbonate, pH 10 (C. van der Does, unpublished results) and it seems, therefore, that a significant fraction of the membrane-bound SecA in the overproducing strain is firmly associated with the SecYEG complex. This form of SecA is catalytically active, as urea-treated membranes retain a significant level of translocation ATPase and translocation activity.

SecA penetrates the cytoplasmic membrane with a domain that is periplasmically exposed (Kim *et al.*, 1994). This inserted state has been detected under conditions in which the *secDF* locus is overproduced (Gardel *et al.*, 1990), while the translocase is not actively involved in protein translocation. The present study shows that the functional overproduction of the SecYEG protein results in a larger fraction of membrane-bound SecA being accessible to trypsin in RSO membrane vesicles. In contrast to the study of Kim *et al.* (1994), this study indicates that SecA is not completely digested but only truncated to a 98 kDa tryptic fragment. Two independent techniques were used to determine whether trypsin cleaves the SecA at the carboxyl or amino terminus. In contrast to intact SecA, the 98 kDa fragment could not be immunostained by an anti-peptide antibody that recognizes the carboxy-terminal 20 amino acids of SecA, and could not be alkylated by biotin-maleimide that reacts with the cysteine residues present in the carboxyl terminus of SecA. Therefore, these data suggest that the SecYEG-associated SecA protrudes from the cytoplasmic membrane with a carboxy-terminal domain. This region has been implicated in membrane binding before (Breukink *et al.*, 1995), but not in the context of the SecYEG-complex. It also seems to be involved in coupling of ATP hydrolysis to protein translocation, and in the interaction between SecA and SecB (Breukink *et al.*, 1995; Rajapandi and Oliver, 1994; P. Fekkes, unpublished results). Although the carboxy-terminal region is not essential for SecA activity *per se*, it is essential for growth, and its removal interferes with *in vitro* translocation.

It has been shown that a membrane-protected tryptic 30 kDa fragment of SecA is formed when the protein is actively involved in translocation (Economou and Wickner, 1994), or when it is supplied with a non-hydrolysable ATP analogue such as AMP–PNP (Economou *et al.*, 1995). Overexpression of SecD and SecF stabilizes the membrane-inserted tryptic 30 kDa SecA fragment at the SecYEG complex (Economou *et al.*, 1995). In native membrane vesicles, the carboxyl terminus of SecA protrudes from the periplasmic side of the membrane (this study). Mechanistically, membrane penetration of the carboxyl terminus of SecA may serve to anchor the protein stably at the SecYEG complex. This may allow an efficient ATP-driven movement (i.e. the creation of a 'pushing' force) of another SecA domain into the membrane concurrently with a preprotein segment (Economou and Wickner, 1994) driving the stepwise translocation of the preprotein. Such a model would explain why truncation of the carboxyl terminus results in a poorer coupling of ATP hydrolysis to preprotein translocation (Breukink *et al.*, 1995; Rajapandi and Oliver, 1994) without losing the ability to support translocation.

In conclusion, the current study suggests that SecA is an intrinsic subunit of the translocase complex and protrudes



from the membrane while it is associated with the SecYEG complex. In this respect, it seems to resemble the ATPase domains of binding protein-dependent transport systems that stably penetrate the membrane while in association with the integral membrane subunits (Baichwal *et al.*, 1993). Unlike these ATPase subunits, SecA is a dissociable subunit (Van der Wolk *et al.*, 1993; Economou and Wickner, 1994). It will be important to define the molecular details of SecA membrane insertion, how the different domains and subunits of the SecA dimer are involved in this process, and how this process is linked to preprotein translocation.

## Experimental procedures

### Materials

*E. coli* SecA (Cabelli *et al.*, 1988; Cunningham *et al.*, 1989), SecB (Weiss *et al.*, 1988), and proOmpA (Crooke *et al.*, 1988) were purified as described. Purified proOmpA was labelled with carrier-free  $^{125}\text{I}$  (Radiochemical Centre) to specific activity of about  $4 \times 10^4$  cpm  $\mu\text{mol}^{-1}$  according to the following procedure. Purified proOmpA (100  $\mu\text{g}$ ) was suspended in 200  $\mu\text{l}$  of Buffer A (50 mM Tris-HCl, pH 7.6, 6 M urea, 50 mM KCl, 5 mM  $\text{MgCl}_2$ ), and transferred to a reaction vial coated with IODO-GEN Iodination Reagent (Pierce). The reaction was started by the addition of 2  $\mu\text{l}$  of  $\text{K}^{125}\text{I}$  (200  $\mu\text{Ci}$ ), incubation for 15 min at room temperature, and termination by transferring the mixture into a new reaction vial containing DTT at a final concentration of 10 mM. Free iodine was removed by chromatography on a PD-10 Sephadex column (Pharmacia Biotech AB) which was prewashed with Buffer A containing 1 mM DTT.

SecA polyclonal antiserum (pAb) 1045 (Neosystem Laboratoire) was raised against the synthetic peptide  $\text{H}_2\text{N-GR-NDPSPCGSGKQKQCHGR-COOH}$ , and recognizes amino acid residues 879–901 of SecA. The mouse oAb is a mixture of monoclonal antibodies (mAbs) raised against different epitopes of *E. coli* SecA (T. den Blaauwen, unpublished results). pAbs against synthetic peptides corresponding to amino acid residues 64–81 of SecE ( $\text{H}_2\text{N-KGKATVAFAREARTEVRK}$ ) and 88–97 of SecG ( $\text{H}_2\text{N-APAKTEQTQP}$ ) were raised by Research Genetics Inc. pAbs directed against SecY, SecA and SecB were from W. Wickner (Dartmouth College, Hanover, NH, USA), the pAb against the cytosolic  $\text{F}_1$  domain of the *E. coli* ATPase was from K.-H. Altendorf (Universität Osnabrück, Osnabrück, Germany), and the mAb F2-12 against PBP1B was from N. Nanninga (University of Amsterdam, Amsterdam, The Netherlands). Trypsin (specific activity of 11 000 U  $\text{mg}^{-1}$  protein) and soybean trypsin inhibitor were purchased from Sigma Chemical Co. Proteinase K was from Boehringer Mannheim, and MPB was from Molecular Probes.

### Bacterial strains and growth conditions

The strains used in this work are shown in Table 1. Unless indicated otherwise, strains were grown aerobically at 37°C on L broth supplemented with 50  $\mu\text{g}$  of ampicillin  $\text{ml}^{-1}$  until the end of the logarithmic phase. For the induction of plasmid-encoded genes under the control of an IPTG-inducible

promoter, exponentially growing cultures were supplemented with 0.5 mM IPTG at  $\text{OD}_{660} \approx 0.5$  and grown for another 2 h.

### Construction of a synthetic operon containing the secY, secE and secG genes

To construct a synthetic *secYEG* operon, individual genes were amplified from *E. coli* chromosomal DNA by the polymerase chain reaction (PCR), using the primers described in Table 3. All PCR reactions were performed with *Pwo* polymerase (Boehringer Mannheim) using a Biometra trio-thermoblock, employing the manufacturers' recommendations. The PCR products contained the entire genes with new ribosome-binding sites (RBS), and new restriction sites directly in front of the RBS or directly after the stop codon. In addition, new *NcoI* sites were created in the start codons of *secY* and *secE* to facilitate the cloning in various expression vectors. The introduction of the *NcoI* site in *secE* caused a substitution of the serine at position 2 by a glycine. The *secY* gene contains an internal *Clal* site. To simplify cloning procedures, this *Clal* site was removed by PCR using the *secYΔClal* and the *secY*, reverse primer. PCR fragments were digested with *KpnI/Clal* (*secY*), *Clal/BamHI* (*secE*), *BamHI/XbaI* (*secG*), and *SalI/Clal* (*secYΔClal*), cloned in pBluescript-II SK+ (Stratagene) and subsequently sequenced on a Vistra DNA sequencer 725 (Amersham) using the automated Δtaq sequencing kit of Amersham. A synthetic operon of *secY–secE–secG* under the control of the *T7* promoter in pBluescript-II SK+ was derived from the single gene constructs by standard cloning techniques (see Table 1) essentially as described by Sambrook *et al.* (1989). To place the synthetic *secYEG* operon under the control of the *trc* promoter, the partially digested large *NcoI/XbaI* fragment from pET338 was cloned in pET324 digested with *NcoI/XbaI* to yield pET340. The pET324 vector was derived from pTRC99A (Amann *et al.*,

**Table 3.** Mismatch oligonucleotides and PCR amplification primers

Primer	Sequence
<b>Mismatch oligonucleotide</b>	
<i>secYΔClal</i>	5'-CGGCGAAGTAGTCGACAAAGTAATG SalI
<b>PCR amplification primers</b>	
<i>secY</i> , forward	5'-CGCGGTACCAGGAGTAGCCCATGGCTAAAC KpnI NcoI
<i>secY</i> , reverse	5'-GGCATCGATTATCGGCCGTAGCC Clal
<i>secE</i> , forward	5'-GCGATCGATAGGAAACAGACCATGGGTGCGA Clal NcoI
<i>secE</i> , reverse	5'-GCGGATCCTTAGAACCTCAGGCCAGTG BamHI
<i>secG</i> , forward	5'-GCGGATCCAGGAGTTGATTATGTATGAAGCTC BamHI
<i>secG</i> , reverse	5'-CGTCTAGATTAGTTCCGGGATATCGC XbaI

Recognition sites of indicated restriction enzymes are underlined. Introduced ribosome-binding sites, start and stop codons are indicated in bold.

1988). The *lacZ $\alpha$*  gene from pUC19 (Yanisch-Perron *et al.*, 1985) was cloned into the multiple cloning site of pTRC99A to obtain pET324, which allows blue/white screening on IPTG/Xgal plates.

#### *Overexpression of SecY, SecE, and SecG, subcellular fractionation, and immunoblotting*

Cells overexpressing SecY, SecE, and SecG were grown as described above, harvested by centrifugation, washed once with 20% glycerol, 50 mM Tris-HCl, pH 8.0, and resuspended in the same buffer. The cell suspension (20 mg of protein ml<sup>-1</sup>) was sonicated on ice with a Soniprep 150 (MSE Instruments) using 30 pulses of 15 s duration at 6 microns. Cell debris was removed by low-speed centrifugation (Eppendorf, 4000 × g, 5 min), and the supernatant (lysate) was fractionated in a soluble (cytoplasmic) and in a pellet (membrane) fraction by ultracentrifugation (TLA100, 200 000 × g, 60 min). Pellet fractions were resuspended in 50 mM NaPi, pH 7.0, in a volume identical to that of the original cell lysate. Equal volumes of the fractions were analysed on 10 or 15% SDS–PAGE (Laemmli, 1970) stained with Coomassie brilliant blue or blotted on PVDF membranes (Millipore) using a semi-dry blotter (Bio-Rad). Immunodetection was carried out with pAb raised against synthetic peptides corresponding to regions of SecY, SecE, and SecG, and a pAb raised against SecA. Blots were developed using a chemiluminescence kit (Tropix).

#### *Isolation of membrane vesicles and trypsin digestion*

ISO and RSO membrane vesicles of *E. coli* SF100 cells were isolated as described by Kaback (1971) and Poolman *et al.* (1983), respectively, and stored in liquid N<sub>2</sub>. Vesicles (0.65 mg protein ml<sup>-1</sup>; final volume 50 µl) were incubated with various trypsin concentrations (0.0022–1.8 mg ml<sup>-1</sup>) for 15 min on ice for SecA and PBP1B, and for 3 h at 30°C for the cytosolic F<sub>1</sub>–β subunit of the *E. coli* F<sub>1</sub>F<sub>0</sub>–ATPase. The proteolysis was stopped by the addition of soybean trypsin inhibitor (5 µl of a 10 mg ml<sup>-1</sup> solution), and 10 µl (about 6 µg of membrane protein) of this suspension was loaded on 10% SDS–PAGE. In the case of SecA digestion, the samples were normalized with respect to the amount of SecA present in the membranes prior to loading on the gels. Immunoblotting and detection were carried out with a pAb against SecA, a pAb against the subunit of the F<sub>1</sub>–domain, and mAb F2-12 against PBP1B (den Blaauwen and Nanninga, 1990). Blots were developed with alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgG (Sigma) and bromo-4-chloro-3-indolyl phosphate/p-nitro blue tetrazolium or a chemiluminescence kit (Tropix). Films were densitometrically scanned using a Dextra DF-2400T scanner (Dextra Technology Corporation) and analysed using SigmaScan/Image (Jandel Corp.).

#### *Maleimidopropionyl-biotinylation (MPB) of SecA*

The sulphhydryl groups of SecA in RSO membrane vesicles (0.65 mg protein ml<sup>-1</sup>), which were incubated without or with trypsin (9 µg ml<sup>-1</sup>), were alkylated with the membrane-permeable maleimide MPB (Bayer *et al.*, 1985; Loo and Clarke, 1995) as follows: before trypsin digestion, the cysteines of

SecA were reduced in 50 mM Tris-HCl, pH 7.6, 0.1 mM DTT and incubated for 30 min on ice. Prior to alkylation by 0.8 mM MPB for 30 min on ice, the DTT was diluted to 0.015 mM by the addition of 50 mM Tris-HCl, pH 7.6, and the samples were sonicated to rapidly distribute the MPB across the membrane. The reaction was quenched by incubation for 20 min on ice in the presence of 80 mM DTT. Samples were immunoprecipitated with a pAb against SecA and analysed by SDS–PAGE. Immunoblotting and detection were carried out with an oAb against SecA and on an identical immunoblot with a *Streptococcus* avidin–alkaline phosphate conjugate (Boehringer Mannheim) using the chemiluminescence kit.

#### *In vitro translocation and SecA translocation ATPase assays*

*In vitro* translocation of <sup>125</sup>I-labelled proOmpA into inner membrane vesicles of *E. coli* vesicles was assayed by its accessibility to added proteinase K (Cunningham *et al.*, 1989). Reaction mixtures (50 µl) contained the following: 50 mM HEPES–KOH, pH 7.5, 30 mM KCl, 0.5 mg ml<sup>-1</sup> BSA, 2 mM DTT, and 2 mM Mg(OAc)<sub>2</sub>, 0.5 µg of SecA protein, 1.6 µg of SecB protein, 2 mM ATP, inner membranes (15 µg of protein), 10 mM creatine phosphate, and 0.5 µg of creatine kinase. Reactions were initiated by the addition of 1 µl of <sup>125</sup>I-labelled proOmpA (0.4 µg) and translocation followed at 37°C. Samples were treated with proteinase K (0.1 mg ml<sup>-1</sup>) for 15 min on ice, precipitated with 7.5% (w/v) TCA, washed with acetone, and solubilized in SDS–sample buffer. Samples were separated by 10% SDS–PAGE and analysed by autoradiography.

Translocation ATPase of 6 M urea-extracted membranes (Cunningham *et al.*, 1989) was measured as described by Lill *et al.* (1989) except that the reactions (50 µl final volume) were supplemented with 1.6 µg of SecB.

#### *Other analytical techniques*

The protein concentration of whole cells was determined by the method of Lowry (Lowry *et al.*, 1951) in the presence of SDS, using BSA as a standard. Phospholipids were chloroform/methanol-extracted according to Blight and Dyer (1959) and Ames (1968), and stored at –20°C under N<sub>2</sub> in chloroform/methanol (1:2 v/v). Inorganic phosphate was determined using the method of Rousser *et al.* (1970). Phospholipids (700 nmol of inorganic phosphate) were spotted onto pre-coated silica-gel plates (Merck, Kieselgel 60) and separated by two-dimensional, thin-layer chromatography using the following solvent systems: A. chloroform/methanol/ammonia/water (90:54:5.5:5, v/v), and B. chloroform/methanol/acetic acid/water (90:40:12:2, v/v). For the identification of the phospholipid species, chromatograms were stained with I<sub>2</sub> vapour (lipids) and ninhydrin (amino groups), and compared with the chromatographic behaviour of phospholipid standards. Phospholipids were stained by the method of Dittmer and Lester (1964), excised from the silica gel, and quantified by determination of phosphorous content.

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